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INVOLVEMENT OF AN NAD(P)H OXIDASE IN THE ELICITOR-INDUCIBLE OXIDATIVE BURST OF SOYBEAN

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Key Word Index—*Glycine max*; Fabaceae; β-glucan elicitor; oxidative burst; NAD(P)H oxidase; glyceollins.

Abstract—The production of H_2O_2 in soybean (*Glycine max* L. cv Harosoy 63) suspension cells upon treatment with a 1,3-1,6-β-glucan elicitor fraction from the cell walls of *Phytophthora sojae* was investigated. The β-glucan elicitor used in these studies was specifically recognized by the soybean β-glucan-binding proteins and induced phytoalexin synthesis in the cell culture. Production of H_2O_2 was measured by the *in situ* oxidation of *o*-dianisidine and was both a time- and a concentration-dependent process, with a maximal response occurring after 20–25 min. Inhibitor studies showed that catalase, peroxidase inhibitors and ascorbate abolished the H_2O_2-mediated *o*-dianisidine oxidation. Diphenyleneiodonium, but not *p*-hydroxymercury benzoic acid, both known inhibitors of the mammalian NADPH oxidase involved in the oxidative burst of phagocytes, was a strong inhibitor of the inducible H_2O_2 production with a concentration for 50% inhibition of 4 μM. The inhibitor studies also indicated that the oxidase might be at least in part responsible for H_2O_2 production by the cells. Furthermore, diphenyleneiodonium was the only inhibitor of O_2^- synthesis in cell-free analyses with a concentration for 50% inhibition of 2.2 μM. Neither ascorbate nor diphenyleneiodonium were able to inhibit elicitor-induced phytoalexin accumulation, indicating that in soybean H_2O_2 and O_2^- may not be directly responsible for phytoalexin production, but may be an independent defence response.

INTRODUCTION

Inducible plant defence responses can be activated upon infection with pathogens or following treatment with elicitors [1]. These responses include specific gene activation, as in the case of phytoalexin accumulation [2] and of various defence-related genes [3]. In addition to these long-lasting reactions, a rapid and localized defence response, the so-called oxidative burst, is elicited. The latter is characterized by the production of reactive oxygen species such as H_2O_2 and O_2^- within minutes at the site of infection [4, 5]. These oxygen species may have direct antimicrobial effects as well as toxic effects on the host tissue [6]. Reactive oxygen species produced in response to pathogens and elicitors have also been suspected to have a role in other defence mechanisms, including lipid peroxidation, lignin formation, hypersensitive response and phytoalexin production [5, 7]. This latter putative role remains, however, a controversial issue [7].

A number of recent reports postulated the involvement of a NADPH oxidase, as known for phagocytes [8], besides other enzymes such as peroxidases or lipoxygenases [7], in the oxidative burst of plants [5, 9–11]. Reactive oxygen species were observed in different plants after challenge with pathogens [10, 12] or treatment with biotic elicitors [9, 10, 12–16]. Several of these investigations were done with soybean (*Glycine max* L.) cell suspension cultures [9, 10, 13–15], although different soybean cultivars and different elicitors were used. The plant enzyme(s) involved in the production of reactive oxygen species is, however, not well characterized [5].

The interaction between soybean and its fungal pathogen *Phytophthora sojae* has been thoroughly studied in recent years. The structure of an elicitor-active oligosaccharide derived from the fungal cell walls was characterized as 1,3-1,6-β-glucan [17, 18]. High affinity β-glucan-binding proteins, that might be involved in signal perception leading to an elicitor-induced phytoalexin response in soybean, were identified and characterized [19–24]. Transduction of the elicitor signal appears to require rapid and transient changes of ion fluxes across the plasma membrane of soybean cells [25].

This well characterized soybean-β-glucan elicitor system was rarely used in studies on the oxidative

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burst. Lindner *et al.* [13] demonstrated, in a preliminary examination, the ability of the cell culture to respond to β-glucan elicitor treatment with the production of reactive oxygen species. Levine *et al.* [10] suspected that H_2O_2 might be involved as a local trigger in programmed cell death. Furthermore, H_2O_2 in the latter work was reported to function as a diffusible signal in the induction of cellular protectant genes, but not in the induction of defence-related genes encoding early enzymes of the phytoalexin synthesis.

In an effort to better understand the generation of active oxygen species, as well as the role of the oxidative burst in soybean upon treatment with the fungal β-glucan elicitor, we characterized more extensively the production of H_2O_2 and O_2^- *in situ* and *in vitro*, respectively. The studies disclosed ligand-binding and phytoalexin-inducing properties of the β-glucan elicitor in the soybean cell culture similar to those found in other soybean tissues. This correlation offered the possibility to investigate the relationship between the oxidative burst and phytoalexin production in the same experimental system.

RESULTS

Analysis of the β-glucan elicitor activities

In earlier studies it was demonstrated that a radioactively labelled fungal β-glucan fraction bound to soybean protoplasts derived from suspension-cultured cells [26]. In order to confirm that β-glucan-binding sites were present in the same plant tissue that was also used for measuring biological responses to the β-glucan elicitor, comparative assays on β-glucan binding and stimulation of phytoalexin production were performed. The ability of the β-glucan fraction to compete with the radioligand, ^{125}I-labelled 2-(4-aminophenyl)ethylamine conjugate of the hepta-β-glucoside (HG-APEA), for the microsomal β-glucan-binding proteins from cell cultures was tested in an assay that was described for binding studies on root membranes [20]. The concentration of the competing β-glucan giving 50% inhibition of binding of the radiolabeled ligand was found to be about 53 ng per assay, corresponding to 0.26 μg ml^{-1}. These results indicated a high-affinity interaction between binding sites and β-glucan, similar to that reported for other soybean tissues [20, 21]. The β-glucan fraction was also capable of stimulating phytoalexin accumulation in the cell culture. The level of the soybean phytoalexins, glyceollins, was found to be 8.1 ± 5.6 nmol per g fresh mass in untreated cells and 49.0 ± 20.0 ($n = 7$) nmol per g fresh mass in elicitor treated cells (200 μg ml^{-1}), respectively. The total amount of glyceollins was to some extent variable in different experiments, possibly due to changes in the responsiveness of the cell cultures during extended periods of subculturing. The ratio of phytoalexin levels of elicited vs non-elicited cells, however, remained very similar (around 6:1).

Investigation of hydrogen peroxide and superoxide anion production

The *in situ* production of H_2O_2 is commonly measured by using fluorescent compounds [9, 10, 13]. In the present investigations, we used the colourless *o*-dianisidine which was oxidized with H_2O_2 as substrate by endogenous peroxidase activity to give a photometrically measurable compound [27]. The specific activity of the peroxidase was determined to be 44 ± 4.0 nkat mg protein^{-1}. Neither elicitor (50 nkat mg protein^{-1}) nor any other compound used in this study affected the activity of this enzyme with the exception of the known peroxidase inhibitors KCN and SHAM (data not shown). H_2O_2 alone was not able to oxidize *o*-dianisidine. The elicitor-inducible production of H_2O_2 in soybean cell cultures was a time-dependent, transient process with an apparent lag of about 5 min and a full response after 20–25 min [Fig. 1(A)]. The cells showed increasing H_2O_2 production in response to increasing concentration of β-glucan elicitor [Fig. 1(B)]. The elicitor concentration yielding 50% of the maximal production was about 7.8 μg ml^{-1}. To confirm that the reaction with *o*-dianisidine as substrate was indeed dependent on H_2O_2 production a H_2O_2-destroying catalase was added. This enzyme (133.4 μkat ml^{-1}) completely abolished the increase in *A* at 450 nm (Table 1). The same result was obtained by treatment with KCN, while SHAM was only partially effective at the concentration used (Table 1), confirming that the reaction between H_2O_2 and *o*-dianisidine was dependent on an endogenous peroxidase activity. Conversely, none of these inhibitors showed any effect on O_2^- synthesis *in vitro* (Table 1). Furthermore, neither the translation inhibitor cycloheximide nor the transcription inhibitor actinomycin D (Table 1) had any influence on the elicitor-mediated H_2O_2 generation, indicating that this rapid defence response is independent of *de novo* protein synthesis. The ineffectiveness of actinomycin D on H_2O_2 production (Table 1) also indicted that an auxin-stimulated NADH oxidase, that is present in soybean hypocotyls [28], was not involved in the oxidative burst of the cell culture.

Because O_2^- represents an intermediate red–ox state between O_2 and H_2O_2 [4, 5], inhibitors of the O_2^--generating processes were tested. The O_2^--producing enzyme in phagocytes, NADPH oxidase, is specifically inhibited by *p*-hydroxymercury benzoic acid (*p*-HMB) and diphenyleneiodonium (DPI) [8]. In soybean, *p*-HMB only slightly affected elicitor-induced H_2O_2 production up to a concentration of 100 μM *in situ* (Table 1). Concentrations higher than 100 μM could not be used as they affected binding of the β-glucan elicitor to the binding protein (data not shown). In contrast, DPI inhibited H_2O_2 generation in the soybean cell culture up to 65% in the concentration range between 1 and 100 μM (Fig. 2). In this range neither the elicitor binding *in vitro* nor the activity of the endogenous peroxidase were affected. The specific activity of the

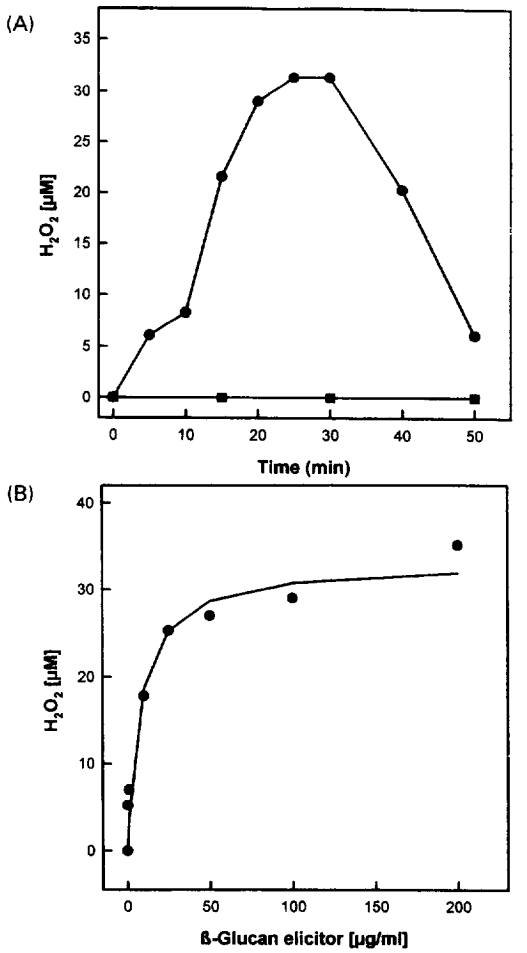

Fig. 1. (A) Time course for the accumulation of H_2O_2 in soybean cells after treatment with β-glucan elicitor and in untreated control cells. At time 0, either elicitor at 50 μg ml^{-1} (●) or H_2O (■) was added. *o*-Dianisidine was added directly before measuring absorbance at times indicated. The production of H_2O_2 was monitored in the culture medium by measuring the absorbance (A) at 450 nm. All values were corrected for the basic absorbance obtained at time 0, representing 7.0 μM H_2O_2. (B) Dependence of H_2O_2 production in soybean cell cultures on the concentration of β-glucan elicitor. The production of H_2O_2 was monitored in the culture medium after 20 min of incubation with elicitor. Soybean cells (0.8 g fr. wt) were incubated in 4 ml medium containing 50 μM of *o*-dianisidine. All values were corrected for the basic absorbance.

Table 1. Effect of putative inhibitors on β-glucan elicitor-induced production of H_2O_2 *in situ* and O_2^- production *in vitro* in soybean cell cultures

Treatment	Percent of control	
	in vitro	*in situ*
	%	
None	100	100
Catalase (46.7 μkat ml^{-1})	n.d.	56
Catalase (133.4 μkat ml^{-1})	n.d.	0
SHAM (1 mM)	95	37
KCN (0.2 mM)	163	0
Cycloheximide (20 μM)	n.d.	96
Actinomycin D (0.1 μM)	96	107
p-HMB (0.1 mM)	77	85

SHAM, salicylhydroxamic acid; *p*-HMB, *p*-hydroxymercury benzoic acid; n.d., not determined.

In situ the production of H_2O_2 was monitored in the supernatant by measuring the absorbance (A) at 450 nm after 20 min of incubation with 50 μg ml^{-1} β-glucan elicitor. The maximum elicitor-inducible H_2O_2 concentration was 37($\pm$6) μM (n = 3).

In vitro the production of O_2^- was monitored by measuring the absorbance at 570 nm over 5 min. The maximum activity of the O_2^- synthase was 0.23 ($\pm$0.03) nkat mg protein^{-1} (n = 3).

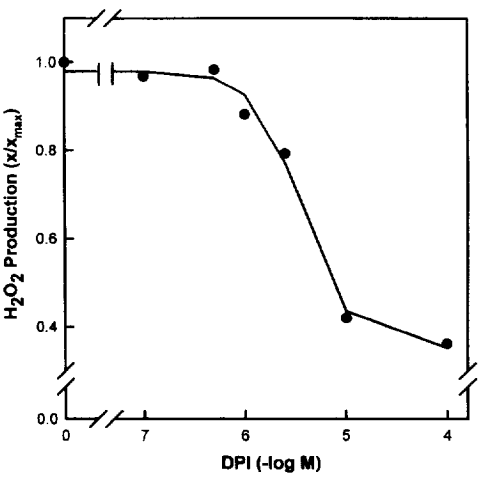

Fig. 2. Effects of diphenyleneiodonium (DPI) on H_2O_2 production in soybean cell cultures treated with a fixed concentration of β-glucan elicitor (50 μg ml^{-1}). Soybean cells (0.8 g fr. wt) were incubated in 4 ml medium containing 50 μM of *o*-dianisidine. The production of H_2O_2 was monitored in the culture medium by measuring the absorbance (A) at 450 nm after 20 min of incubation with elicitor. The response of the soybean cells was calculated as A/A_{max}. A_{max} represents 44$\pm$6 μM H_2O_2 obtained with 50 μg ml^{-1} β-glucan elicitor (n = 3). All values were corrected for the basic absorbance.

endogenous peroxidase was determined to be 43 nkat mg protein^{-1} in the presence of 100 μM DPI, a value being very similar to that in the absence of DPI (see above). Again, *p*-HMB only slightly affected the O_2^--dependent increase in A at 570 nm (Table 1). Treatment with DPI, however, showed strong inhibition of O_2^- production *in vitro* (Fig. 3). When DPI was added together with NADPH, an IC_{50} value of 140 μM was found. The effectiveness of DPI was 64 times higher (IC_{50} of 2.2 μM) if the assay was preincubated for 10 min with 20 μM of NADPH, prior to addition of DPI.

O_2^- synthesis was strongly dependent on the presence of NADPH but NADH was also accepted as substrate. The kinetics of the reaction showed that the K_m value for NADPH (14.3 μM) was around three times lower than that for NADH (44.5 μM), with a specific activity in the presence of NADPH of 0.23$\pm$0.02 nkat mg protein^{-1} and 0.42$\pm$0.16 nkat mg protein^{-1} in the presence of NADH.

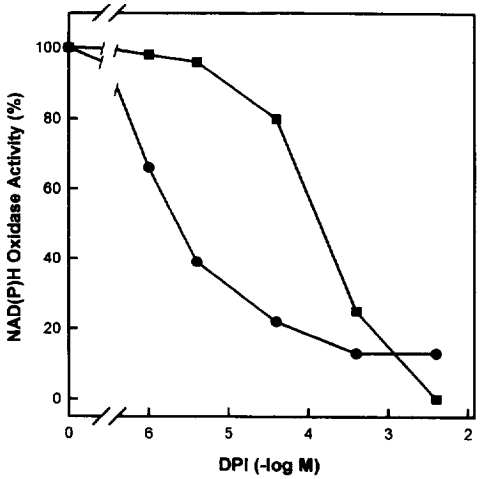

Fig. 3. Effect of increasing concentrations of diphenyleneiodonium (DPI) on O_2^- production *in vitro*. Oxidase activity with 10 min preincubation (●) of the assay mixture (50 mM KPi, pH 7.5, 0.5 mM $MgCl_2$, 0.02% (v/v) Triton X-100, 0.2 mM NBT, 20 μM NAD(P)H, and 450 μg protein with a ratio of microsomal to cytosolic protein 1:8, in a final volume of 1 ml); activity in the presence of NADPH without preincubation (■). The production of O_2^- was monitored by measuring the absorbance (A) at 570 nm over 5 min of incubation. 100% activity represents 0.20 ± 0.07 nkat mg^{-1} $protein^{-1}$ ($n = 6$). All values were corrected for the basic absorbance.

Table 2. Comparison of the effects of ascorbate and DPI on β-glucan elicitor-induced H_2O_2 and glyceollin production in soybean cell cultures

	H_2O_2		Glyceollin	
Treatment	− β-Glucan	+ β-Glucan	− β-Glucan	+ β-Glucan
		(A/A_{max})		(x/x_{max})
None*	0.34	1.00	0.16	1.00
Ascorbate (10 mM)*	n.d.	0.06	0.74	2.00
None†	0.35	1.00	0.25	1.00
DPI (10 μM)†	n.d.	0.44	0.44	2.80

*†Represent the data of independent experiments; n.d., not determined. DPI, Diphenylene-iodonium.

The production of H_2O_2 was monitored in the supernatant by measuring the absorbance (A) at 450 nm after 20 min of incubation with 50 μg ml^{-1} β-glucan elicitor. The response of the soybean cells was calculated as A/A_{max}, where A_{max} represents 40 (±14) μM H_2O_2 ($n = 6$). For glyceollin determinations soybean cell cultures were treated either with β-glucan elicitor (200 μg ml^{-1}) or with water. After 48 hr glyceollin was extracted from the medium and analysed as described in the Experimental. The response of the soybean cells was calculated as x/x_{max}, where x_{max} represents 49.0 (±20.0) nmol glyceollin per g fresh mass ($n = 7$).

Influence of inhibitors and scavengers on phytoalexin accumulation

One putative role that H_2O_2 and O_2^- may play during plant–pathogen interactions is that they could serve as signal intermediates in the activation of defence responses [5]. To investigate this possibility, we determined the influence of ascorbate and DPI on β-glucan elicitor-mediated glyceollin accumulation. At concentrations where these compounds showed maximal effects on the elicitor-induced H_2O_2 production, no inhibition of phytoalexin accumulation was observed (Table 2). In the absence of β-glucan elicitor the amount of glyceollins was enhanced 1.8- to 4.6-fold only by the addition of these substances. In the presence of elicitor the accumulation of glyceollins further increased 2- to 2.8-fold upon simultaneous treatment with either the inhibitor of the O_2^- generating NADPH oxidase, DPI, or the H_2O_2 scavenger ascorbate.

DISCUSSION

Active oxygen species may potentially affect various cellular processes in plant–pathogen interactions. Many recent investigations have, therefore, focused on the origin, nature, and role of these oxygen species during early reactions of plants to infection or elicitor treatment. For the detection of active oxygen species in the present studies, an assay has been used that measures the peroxidase-catalysed oxidation of *o*-dianisidine [27]. That the oxidation of *o*-dianisidine was indeed dependent on H_2O_2 was confirmed by the inhibitory action of added catalase, and by the inhibition of dye oxidation when the endogenous peroxidase activity was inhibited specifically (Table 1). No influence of either elicitor or other compounds on the level of endogenous peroxidase activity was detectable. This is in contrast to the results of Dwyer *et al.* [29], who reported the DPI-dependent inhibition of the peroxidase catalysed oxidization of pyranine by H_2O_2. One explanation for this seeming discrepancy between the results may be that the peroxidases involved in these reactions are quite different.

The β-glucan elicitor from *P. sojae* induced a rapid production of H_2O_2 in soybean cell cultures. Both the apparent lag time between elicitor addition and onset of H_2O_2 production, and the time course, but not the extent of H_2O_2 accumulation, was independent of the elicitor concentration applied. These results were similar to those reported for the effects of other elicitors on soybean cells (e.g. oligogalacturonic acid, [10, 14, 15]; *Verticillium dahliae* 277 elicitor, [9, 14]). They also resemble the immediate, relatively short-lived response of cell cultures from several plants, including soybean, after the addition of either incompatible or compatible bacteria [7]. The elicitor concentration, that was required for half-maximal induction of H_2O_2 production [Fig. 1(B)], was about 30 times higher than the concentration of elicitor that was giving 50% inhibition of binding of the radiolabelled heptaglucoside elicitor to the soybean membrane fraction. This difference has been also repeatedly found when the

concentration dependence for elicitor binding and phytoalexin induction was compared [20, 30]. This finding could indicate that a relatively high occupancy of the binding sites is required for initiating cellular responses by the β-glucan elicitors [30]. Concomitant with rapid H_2O_2 production are changes in Ca^{2+}, H^+, and Cl^- fluxes across the plasma membrane of soybean cells [25]. All of these responses precede the earliest detectable reactions associated with the activation of genes that are involved in phytoalexin production [31, 32].

Elicitors are useful tools in studies concerning the mechanism(s) by which pathogens trigger active oxygen production and the likely sources for their production. Several recent reports suggested the involvement of a NADPH oxidase that may be similar to an enzyme present in phagocytes [5, 10]. The respiratory burst in phagocytes is attributed to an NADPH oxidase that transfers electrons from NADPH on the internal side of the plasma membrane to molecular oxygen on the external side of the membrane, leading to the production of O_2^- [33]. The superoxide is either spontaneously, or, in the reaction catalysed by superoxide dismutase reduced to H_2O_2. The only indirect evidence for the existence of a NADPH oxidase in plants is the inhibition of the elicitor-induced H_2O_2 production by DPI, the specific inhibitor of the mammalian NADPH oxidase [10, 16]. In this study we also detected an inhibition in the micromolar range by DPI of β-glucan-elicited H_2O_2 accumulation (Fig. 2), but the inhibitor only partially affected H_2O_2 production as in the case of rose cells [16]. The residual *o*-dianisidine-oxidizing activity could be completely abolished by catalase, indicating that some other O_2^-- or H_2O_2-generating process was present, which could not be inhibited by DPI. The only compound showing a total inhibition of the *o*-dianisidine-oxidizing activity was KCN (Table 1). This result suggests the possible participation of an additional peroxidase in the generation of H_2O_2 [7], which again is DPI-insensitive. A further inhibitor of the mammalian NADPH oxidase, *p*-HMB [8], was not active in preventing the formation of active oxygen species in soybean cells (Table 1).

To further confirm the production of O_2^- we used DPI and other inhibitors for cell-free analyses (Table 1, Fig. 3). All other inhibitors tested in this study, including peroxidase inhibitors, failed to inhibit O_2^- production. This indicates that the DPI-sensitive activity is not a peroxidase and is different from that postulated by Lindner *et al.* [13]. The only earlier investigation of production of O_2^- in a cell-free system used membrane protein extracts from rose cells [34]. These authors showed an IC_{50} value for the inhibition of O_2^- synthesis by DPI in the nanomolar range. In soybean, we determined this value to be 2.2 μM (Fig. 3) which is 20–50 times higher than that reported for extracts from rose cells. Such a discrepancy between the effectiveness of DPI on O_2^- synthesis, which might be due to the state of reduction of the NADPH oxidase, is also known for bovine and human neutrophils [35, 36]. Therefore, the differences between rose and soybean might be not too surprising. The K_m values for the substrates NADPH (14.3 μM) or NADH (44.5 μM) as well as the specific activities of the enzyme in the presence of NADH or NADPH, respectively, are in good agreement with those determined for rose cells [34], indicating that the putative NAD(P)H oxidases in plants possess similar properties. The molecular structure of this enzyme in plants is still unknown. In soybean, only limited information is available from immunological studies that showed recognition of soybean proteins with antisera raised against microsomal [37] or soluble [29] subunits of the mammalian NADPH oxidase. Interestingly, in the case of the soybean cell culture used in the present study, we detected cross reactivity of antisera against the soluble subunits p47 and p67 in the microsomal fraction (data not shown), possibly indicating differences in structure or localization between the NADPH oxidase in plants and mammals.

We also investigated a possible role of reactive oxygen species in the signalling process that activates other defence responses. A connection between the production of reactive oxygen species and of phytoalexins was first suggested by the work of Doke [38, 39], and later analysed in bean [40], soybean [9, 13], white clover [41] and tomato [42]. The effects of the inhibitors, ascorbate and DPI, on the elicitor-mediated phytoalexin production seem to rule out such a suspected role of reactive oxygen species for soybean (Table 2). Glyceollin production was neither inhibited when the O_2^- level was decreased by DPI nor when the H_2O_2 level was decreased by DPI or ascorbate (Fig. 2, Table 2). In the presence of ascorbate peroxidase this enzyme could detoxify H_2O_2 by using ascorbate as a substrate [43, 44]. The presence of an active form of an endogenous ascorbate peroxidase in the soybean cells used was secured (data not shown). Therefore, the observed inhibition of *o*-dianisidine oxidation was very likely due to the elimination of H_2O_2 by adding ascorbate (Table 2). In summary, these results suggest that upon elicitation of soybean cells the generation of O_2^- occurred first, followed by its conversion to H_2O_2. For reasons, as yet unknown, we even found a small enhancement of the glyceollin levels by treatment with these inhibitors. Our results support the conclusions of Levine *et al.* [10], who pointed out that H_2O_2 did not function as a primary signal for the activation of enzymes of phytoalexin biosynthesis in soybean. Also, results with polygalacturonic acid (PGA) as elicitor can be interpreted in the same way. PGA with a degree of polymerization of 12 was most active in eliciting phytoalexin accumulation in soybean [45], whereas PGA with a degree of polymerization of 20 induced only the oxidative burst in soybean cells [14]. Our results, therefore, confirm the theory [5] that in soybean, pathways independent of the oxidative burst contribute to the regulation of phytoalexin accumulation.

EXPERIMENTAL

Chemicals. Diphenyleneiodonium chloride (DPI) was from Alexis Deutschland GmbH, Grünberg, Germany; *p*-hydroxymercury benzoic acid sodium salt (*p*-HMB) from Serva, Heidelberg, Germany; nitroblue tetrazolium (NBT) and cycloheximide from Roth, Karlsruhe, Germany; 3,3′-dimethoxybenzidine dihydrochloride (*o*-dianisidine), salicylhydroxamic acid (SHAM), actinomycin D, and catalase (EC 1.11.1.6) from Sigma.

Plant material and elicitor preparation. Cell suspension cultures of soybean (*Glycine max* L. cv Harosoy 63) were grown as described previously in ref. [46]. Cultures incubated at 24° in the dark on a rotary shaker at 100 rpm and subcultured in fresh medium every 7 days.

Elicitor was prepd by partial acid hydrolysis from purified cell walls of *Phytophthora sojae* (Ps) according to ref. [19]. The branched β-glucan elicitor fr. used in these studies was composed of 420 μg glucose equivalents and 25 μg protein mg^{-1}.

Protein extraction from cell suspension cultures. Microsomal frs were obtained by collecting 5 to 7-day-old soybean cell cultures on a sintered-glass funnel under red. pres. The cells were frozen in liquid N_2, disrupted by using a mortar, and resuspended in buffer using a Potter–Elvehjem-type homogenizer. Buffer used in preps for binding assays contained 50 mM Tris–HCl, pH 7.5, 30 mM $MgCl_2$, 1 mM DTT and 0.2 mM PMSF. After differential centrifugation according to ref. [26] the microsomal pellets were used immediately or stored at −70°.

In preps for superoxide synthase assays the buffer consisted of 50 mM KPi, pH 7.5, 0.5 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 200 mM sucrose and 1 mM PMSF. The homogenate was centrifuged sequentially at 10 000 *g*, 30 min and at 150 000 *g*, 1 hr, to obtain cytosolic and microsomal frs. This latter fr. was resuspended (50 mM KPi, pH 7.5, 0.5 mM $MgCl_2$ and 1 mM PMSF). Both frs were frozen in liquid N_2 and stored at −70° or used directly. Protein content was measured according to ref. [47].

Binding assays. Performed as described in ref. [26]. Microsomal protein (200 μg) was incubated with increasing concs of Ps elicitor in the presence of 3 nM ^{125}I-labelled 2-(4-aminophenyl)ethylamine conjugate of the hepta-β-glucoside (HG-APEA) for 2 hr at 4° in a final vol. of 200 μl binding buffer (25 mM Tris–HCl, pH 8, 100 mM NaCl, 10 mM $MgCl_2$ and 5 mM D-gluconic acid lactone). Synthesis of the HG-APEA was as in ref. [20].

Assay of superoxide anion synthase. The assay was performed according to ref. [48] with some modifications. The standard mixt. contained 50 mM KPi, pH 7.5, 0.5 mM $MgCl_2$, 0.02% (v/v) Triton X-100, 0.2 mM NBT, 20 μM NAD(P)H, and 450 μg protein (ratio of microsomal to cytosolic protein 1:8) in a final vol. of 1 ml. Reaction was started by adding 0.1 ml of a 0.2 mM NAD(P)H soln. Superoxide anion production was determined at 25° by increase in *A* at 570 nm (A_{570}) with time, representing reduction of nitroblue tetrazolium (NBT) [4, 48]. Increase in *A* was linear over the period of 5 min.

Determination of H_2O_2. The assay was performed with 5- to 7-day-old soybean cell suspension cultures. Before treatment the cells were harvested and subcultured for 3 hr in fresh medium without Na-Pi (8 g fresh mass per 40 ml medium). Samples (0.8 g cells in 4 ml medium) were treated, unless otherwise specified, with β-glucan elicitor (50 μg ml^{-1}) at 25° on a rotary shaker at 175 rpm. The cells were allowed to settle for 1 min and portions of the supernatant (1 ml) were assayed for H_2O_2 production. H_2O_2 production was determined by measuring increase in *A* at 450 nm (A_{450}) that resulted from the peroxidase-catalysed oxidation of the non-fluorescent *o*-dianisidine [27], making use of the endogenous peroxidase activity of soybean cells. Relative increase in *A* was calcd from difference of *A* between time of addition of any compound (set as time 0) and time indicated in the individual experiment. Initial concn of the dye *o*-dianisidine was 50 μM. In typical experiments, difference in *A* after 20 min was determined. For control experiments, H_2O was used instead of elicitor soln. Concn of H_2O_2 was calcd from a standard curve obtained by incubating variable amounts of H_2O_2 ranging from 1 to 60 μM, 0.21 μkat horseradish peroxidase (Boehringer) and 50 μM *o*-dianisidine. There was a linear correlation between H_2O_2 concn and A_{450} (data not shown).

Assay of peroxidase activity. Activity of the endogenous peroxidase accepting the substrates *o*-dianisidine and H_2O_2 was determined at 450 nm in a final vol. of 1 ml cell culture medium over a period of 4 min. An aliquot of cell culture supernatant after 3 hr of subculturing, containing 14 μg protein, was used in the assay. Reaction was started by adding *o*-dianisidine and H_2O_2 to give an initial concn of 50 μM and 1 mM, respectively.

Determination of glyceollins. Soybean cell suspension cultures (5-day-old) were subcultured in fresh medium for 15 hr (4 g fresh mass per 40 ml medium). Treatment was done in 8 ml frs of the cell suspension on a rotary shaker at 100 rpm and 24° in the dark. Elicitor concn used in these experiments was 200 μg ml^{-1}. Cells were harvested after 48 hr by filtration under red. pres. on a sintered-glass funnel. The medium was extracted twice with 8 ml EtOAc each. The organic phase was dried over Na_2SO_4 and removed under red. pres. The residue was dissolved in 200 μl EtOH and chromatographed by HPLC (LiChrosorb RP-18, 4 × 250 mm; flow rate 1 ml min^{-1}; linear gradient from 40 to 65% MeOH in 20 min). Compounds were identified and quantified by using ref. substances.

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